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Pathways involved in trifluoperazine-, dibucaine- and praziquantel-induced hemolysis

Sônia Valéria Pinheiro Malheiros, Nilce Correa Meirelles, Eneida de Paula*

Department of Biochemistry, Institute of Biology, State University of Campinas (Unicamp), P.O. Box 6109, CEP 13083-970, Campinas, SP, Brazil

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Abstract

This work elucidates differences in the hemolytic pathway developed by the antipsychotic trifluoperazine (TFP), the local anesthetic dibucaine (DBC) and the antihelminthic praziquantel (PZQ). Their partition coefficients (P) were measured at pH 7.4 between n-octanol, microsomes, liposomes, erythrocyte ghosts and n-octanol/water. The effective drug:lipid molar ratios for the onset of membrane solubilization (Re^{SAT}) and complete hemolysis (Re^{SOL}) were calculated from the experimental P values and compared with a classical surface-active compound treatment [Lichtenberg, D. Biochim. Biophys. Acta 821 (1985) 470–478]. The contribution of charged/uncharged forms of TFP and DBC for the hemolytic activity was also analyzed. In all cases the hemolytic phenomena could be related to the monomeric drug insertion into the membrane. Only for TFP at isosmotic condition lysis occurs at concentrations beyond the CMC of the drug, indicating that micellization facilitates TFP hemolytic effect, while DBC and PZQ reach a real membrane saturation at their monomeric form.

Keywords: Erythrocyte; Surfactant; Membrane; Partition; Ionization; Hemolysis

1. Introduction

Erythrocyte membranes have been extensively studied since the cells can be copiously obtained by venous puncture and the membranes are easily isolated by low-speed centrifugation [1]. Consequently, erythrocytes have become a good model for drug–membrane interaction, providing information concerning, for instance, changes in lipid composition or in cytoskeleton [2], enzymes [3] or other membrane proteins [4].

The information obtained using erythrocytes as a model is especially important when the drugs under study have their site of action at the membrane level, such as antipsychotic agents [5–7] and local anesthetics [8].

* Corresponding author. Fax: +55-19-289-3124.
E-mail address: depaula@unicamp.br (E. de Paula)

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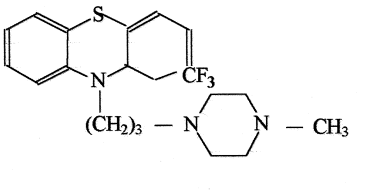
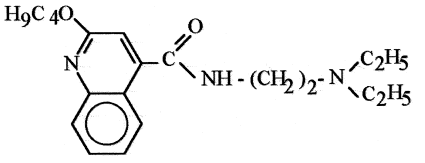
Hemolysis is the disruption of red blood cells which can be caused by the interaction of chemical compounds with the membrane. The hemolytic activity of many amphiphilic substances, including anesthetic, antiinflammatory and neuroleptic drugs have been described by Seeman early in 1966 [9]. According to Kanaho et al. [10], the effect of a drug on the erythrocyte membrane can be attributed to two main phenomena: its insertion into the membrane and the intensity of the membrane-perturbing action of the molecule once incorporated. Once inside the membrane, the drug can occupy specific (protein) and/or non-specific (lipid) binding site(s). Some factors are considered determinant in hemolysis establishment: the hydrophobicity of the compound and the presence of an ionizable group [11].

We have recently published a quantitative study of the interaction of the phenothiazinic antipsychotic agent trifluoperazine (TFP) with the erythrocyte membrane in which we determined the TFP/lipid ratio for erythrocyte membrane protection or lysis [12]. In the present study, we report the interaction of three different drugs with erythrocyte membranes by measuring their partition coefficients between membrane and water and by monitoring their hemolytic activity (protection against or lysis). The comparison of these drugs takes into account the drug/lipid molar ratio to achieve the membrane protection and disruption, as well as the contribution of partitioning, drug micellization and ionization to the hemolytic process. The chosen drugs were TFP, the local anesthetic dibucaine (DBC) and praziquantel (PZQ), an antihelmintic compound (Fig. 1). It is interesting to note that while the interaction of TFP [3,9,11–20] and DBC [9,13,15,17] with erythrocytes has been extensively studied, this is, to our knowledge, the first report concerning PZQ.



TFP



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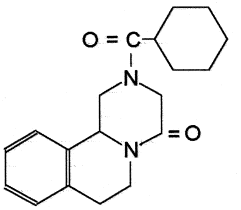

PZQ

Fig. 1. Chemical structure of trifluoperazine (TFP), dibucaine (DBC) and praziquantel (PZQ).

2. Material and methods

TFP hydrochloride, DBC hydrochloride and egg phosphatidylcholine (EPC) were obtained from Sigma Chemical Co., St Louis, MO, USA. Praziquantel was kindly donated by Merck S.A. Indústrias Químicas, São Paulo, SP.

2.1. Membrane preparation

Mouse liver microsomes [20], EPC multilamellar vesicles and mouse erythrocyte membranes were prepared as described previously [12]. Erythrocyte ghost membranes were prepared as described by Dodge et al. [21].

2.2. Protein and phospholipid concentrations

Protein and phospholipid concentrations were determined according to [22,23].

2.3. Partition coefficient (P) determination by phase separation

A known amount of drug was incubated for 10

min, with the membranes kept in PBS (150 mM NaCl, 5 mM sodium phosphate, pH 7.4) at room temperature (22–25°C). The drug concentration remaining in the supernatant after centrifugation at $105\,000 \times g$ for 1 h was optically detected: at 256 nm for TFP ($\varepsilon_M = 23\,500$); at 324 nm ($\varepsilon_M = 3770$) for DBC; and at 260 nm ($\varepsilon_M = 320$) for PZQ, against the respective control (membrane in PBS). Ghosts, rather than whole erythrocytes, were used in partition coefficient determination because the spectrum of hemoglobin released during the experiment overlapped the optical spectra of the drugs, preventing their precise determination in the supernatant. The amount of drug (solute) bound to the apolar phase was obtained by subtracting the supernatant concentration from the total solute concentration measured before phase mixing. The partition coefficient, P, was calculated according to Eq. (1) [24]:

$$P = \frac{n_m(s)/V_m}{n_w(s)/V_w}, \quad (1)$$

where s denotes the solute compound, n is the number of moles of solute, V = volume and the subscripts m and w refer to the membrane and aqueous phase, respectively. The apolar phase volume (V_m) was calculated assuming a lipid density of 1 g/ml for the membranes [12].

2.4. Octanol / water P determination

PBS and *n*-octanol solutions were pre-equilibrated overnight; after drug addition the mixture was vortexed and incubated for 10 min before centrifugation at $260 \times g$, for 5 min. The drug concentration in the water phase was optically determined and P was calculated as described for the phase separation experiments.

2.5. Determination of critical micelle concentration (CMC)

CMC was determined with a K12 Krüss tensiometer. The surface tension of TFP solutions ranging from 0.1 to 100 μM was measured using PBS buffer and the surface tension of DBC solutions ranging from 0.5 to 30 mM was measured using 50 mM phosphate buffer, pH 6.8, both at room temperature.

2.6. Assay of protection against hemolysis

Erythrocytes (0.14% hematocrit) were incubated in hypotonic condition (PBS buffer with 66 mM NaCl, 5 mM phosphate, pH 7.4) to induce 50% hemolysis. Each compound was added in an appropriate concentration range (0.01–100 μM for TFP, 0.001–2.5 mM for DBC and 0.005–5 mM for PZQ) and the samples were incubated for 10, 30 and 60 min. After centrifugation at $260 \times g$ for 3 min, released hemoglobin was measured in the supernatant at 412 nm. The results were expressed on a relative absorbance (RA) scale, with statistical analysis of variance conducted at the SAS program [25], as previously described [12]. RA is the average value of 15 experiments.

2.7. Hemolytic assay

TFP (1–200 μM) and PZQ (0.05–10 mM) were prepared in isotonic PBS. DBC (0.1–10 mM) was prepared in PBS adjusted to pH 6.8. PZQ was dissolved in DMSO before PBS addition so that the final DMSO concentration never exceeded 5% (v/v). Erythrocytes (hematocrit ranging from 0.04 to 0.14%) were added, and the samples kept at room temperature, for 10 min (TFP), 30 min (DBC) and 90 min (PZQ) before centrifugation at $260 \times g$ for 3 min. The different incubation times are due to discrepancies in the time required to induce the maximum effect. Hemoglobin released into the supernatant was detected at 412 nm (for lower hematocrits) and 540 nm (0.14% hematocrit).

The hemolytic effect, measured as percent relative hemolysis (RH), was determined on the basis of released hemoglobin, subtracting the hemolysis obtained for the control (erythrocytes in PBS) from the hemolysis of samples.

2.8. Re (drug / lipid ratio) calculation

Lichtenberg defined C^{SAT} and C^{SOL} as the solute concentration needed for saturation and

total (lipid) membrane solubilization, respectively [26]. In our case, solubilization refers to the operational definition that considers 100% released hemoglobin in the supernatant. C^{SAT} and C^{SOL} were determined in the hemolytic experiments and plotted as a function of erythrocyte membrane lipid concentration, allowing the determination of *Re*, the effective solute/lipid molar ratio both for initial (saturation) and total hemolysis (solubilization), according to Eq. (2) [27,28]:

$$Dt = Re[L + 1/Kb(Re + 1)], \quad (2)$$

where Dt is the total solute (C^{SAT}, C^{SOL}) and L the lipid concentration in the system. *Re* is taken from the slope of the resulting straight line and the *y* intercept corresponds to Dw, the concentration of the free solute in water, equivalent to its CMC [26,29]. Finally K_b (M^{-1}) describes the solute binding to membranes in terms of equilibrium:

$$s + m \rightleftharpoons sm \qquad K_b = \frac{[sm]}{[s][m]}$$

where *s* is the free solute and *sm* is the membrane-forming compound. Binding constants (K_b) and partition coefficients (P) are related through the partial molar volume, ∇, of the lipid phase, according to [8,30]:

$$K_b = P \cdot \nabla. \quad (3)$$

For erythrocytes, ∇ was taken as 0.658 l/mol [12].

3. Results and discussion

Although the drug-induced hemolysis phenomenon has been studied since 1937 [31], up to now, the nature of drug–membrane interaction has not been clearly elucidated. Many authors have described the interaction of amphiphiles with the erythrocyte membranes, reporting the general characteristics of this interaction. For instance, since 1966, Seeman has established that tertiary amines like phenothiazine neuroleptics and local anesthetics induce hemolysis in a biphasic manner [9]; Seeman and coworkers [32,33] reported that many amphiphiles are able to protect erythrocytes against hypotonic hemolysis by intercalating into the membrane, increasing the membrane area/volume ratio of the cell and thereby, the critical hemolytic volume of the erythrocyte. Sheetz and Singer [14] proposed that anionic drugs are echinocytogenic while cationic compounds induce stomatocytosis. Aki and Yamamoto [18] suggested that potent hemolytic drugs such as cationic phenothiazines trigger a thermal effect differing from that of other hemolytic compounds, like anionic antiinflammatories, which induce a slightly negative ΔH. In a recent report we have used microcalorimetry to compare hemolysis induced by TFP, DBC and PZQ. We showed that the heat effect of TFP is four times greater than that of DBC, indicating putative different pathways in hemolytic activity [34]. Since calorimetry is a non-specific technique, the observed heat effect cannot discriminate between any particular events and shows the overall outcome of the hemolytic phenomenon.

Here we intend to analyze the hemolysis caused by these three compounds (TFP, DBC and PZQ) which probably use distinct mechanisms for their membrane-disruption action. Table 1 shows some physicochemical properties of the drugs studied. The phenothiazine derivative TFP and the local anesthetic DBC have an ionizable amine group with a pK of 8.1 and 8.3, respectively [35,36], so that, as discussed further in this paper, both charged (+) and uncharged (:) species are present at physiologic pH. Water solubility is given for both species (Table 1). As amphiphilics, these drugs have a surfactant behavior: they are able to form micelles at the CMC values determined (Table 1). PZQ has a low water solubility and it does not ionize or show any aggregate property.

3.1. Drug partitioning between membranes and water

Table 2 shows TFP, DBC and PZQ partition coefficients (P) between membrane (microsomes, erythrocyte ghosts, liposomes) and water and octanol/water systems, determined at pH 7.4. It can be seen that TFP partition into the membranes is

Table 1
Physicochemical properties of TFP, DBC and PZQ[a]

Drug	MW	Sw (M) Charged form	Sw (M)[a] Uncharged form	CMC (M)
TFP	480.4	1[b]	3.0×10^{-5}	4.2×10^{-5}[d]
DBC	379.9	1.9[c]	3.0×10^{-5}	10.7×10^{-3}[d]
PZQ	312.4	–	1.3×10^{-3}	–

[a] Determined in 20 mM carbonate buffer, pH = 10.5.
[b] In water, according to the Merck Index.
[c] In 20 mM acetate buffer, pH 5.0, according to [40].
[d] Determined in 50 mM PBS at pH 7.4 (TFP) and 6.8 (DBC). Molecular weight (MW), aqueous solubility (Sw) and critical micellar concentration (CMC).

higher than those of DBC and PZQ into any of the membranes. This is curious because TFP is also highly water soluble (see Sw for charged form in Table 1) and should not present the highest partition into membranes at pH 7.4. Differences in ionization, i.e. charged/uncharged drug ratios at physiologic pH, can explain these results. A detailed look at the ionization properties reveals that at pH 7.4 *and in the presence of membranes* TFP is approximately 40% in the uncharged form [12] while DBC is just 16% uncharged (see later in discussion of Eq. (6)). That is why TFP becomes more hydrophobic than DBC at pH 7.4. But what about PZQ? It does not ionize at all around physiologic pH. Why does PZQ show the worst interaction with membranes (Table 2) and — as will be shown further on in the hemolytic experiments — why does it need longer incubation times than TFP and DBC to induce hemolysis? It appears that the low solubility of PZQ determines a non-ideal partitioning (defined by K_b. $S\text{w} < 2$) limiting its entrance into the membrane, as described before for *n*-alcohol series [37–39] and local anesthetics [40]. In this case there is a restraint in membrane partitioning related to the incomplete exclusion of the drugs from the membrane — the 'cutoff' effect — once saturation of the water phase is reached. This non-ideal partitioning of PZQ will also explain the large amounts of the drug and long incubation times required for the hemolytic effect.

Anyway, the high affinity of TFP for biomembranes allows it to easily penetrate the central nervous system and thus to be one of the most potent antipsychotic agents. The stronger binding of TFP to microsomes may reflect some TFP–protein interaction, as discussed before [12]. Table 2 also shows that for each drug used the partition into ghost membranes is lower than the partition into the other bilayers (microsomes and EPC liposomes). To explain the rather small P^{GHOST} values, we must consider the lower fluidity of the ghost membranes, related to the cytoskeleton [42] and high cholesterol content (30% in weight) [41] that may restrict drug partition into them.

The P values obtained for the drugs into different membranes show that the partition varies greatly for each membrane studied. Absolute P values determined between the organic phase (octanol) and water were lower than P values between membranes and water (Table 2); as also reported by other authors [11,13], P^{OCT} values rarely coincide with those found in biomembranes and should be used with caution [12,43].

Even in real anisotropic systems like membranes, our data reveal large differences in P values, depending on the composition of the bilayer and membrane nature (EPC vesicles, microsomes or erythrocyte ghosts). So, we wish to emphasize that when P is used as a hydrophobic parameter it gives an approximate idea, but the nature of the membrane must be always considered, for each apolar/polar partition system.

Table 2
Partition Coefficients[a] for TFP, DBC and PZQ between microsomes (P^{MIC}), erythrocyte ghosts (P^{GHOST}), liposomes (P^{LIP}), octanol (P^{OCT}) and water phase

Drug	P^{MIC}	P^{GHOST}	P^{LIPb}	P^{OCT}
TFP	7172 ± 1229	1380 ± 429	1916 ± 341	452 ± 55
DBC	793 ± 119	375 ± 62	1884 ± 125	265 ± 25
PZQ	488 ± 179	210 ± 52	ND[c]	50 ± 12

[a] Each P value represents the mean ± S.E.M. of nine experiments in 5 mM PBS buffer, pH 7.4, room temperature.
[b] For P^{LIP} we used four freeze-thawing cycles, increasing the incubation time to approximately 30 min.
[c] Not determined due to the high light dispersion that masks PZQ absorbance at 324 nm.

3.2. Hypo- and isotonic drug-induced hemolysis and drug / lipid ratio determination

In a previous work, we showed the hemolytic action of TFP under hypo- and isotonic conditions [12]. A biphasic TFP hemolytic curve obtained for Ht = 0.14% is shown in Fig. 2. TFP — at concentrations up to 17 μM — stabilizes and protects erythrocytes against *hypotonic* lysis, while at higher concentrations — above 17 μM — the upward curvature indicates lysis. The maximal protective TFP concentration (C^{PROT}) is 13 μM, with TFP in its monomer form. The hypotonic curves were performed at three different incubation times, i.e. 10, 30 and 60 min with no significant differences with increasing times, which means that 10 min is enough for TFP to reach the partition equilibrium.

Fig. 2 also shows the biphasic hemolytic effect under *hypotonic conditions* for DBC and PZQ. The protective effect of DBC against hemolysis (inward curvature) is maximum at approximately 120 μM and stabilizes after 30 min of incubation. For PZQ, the concentration and the lower incubation time required to produce a stable protective effect were 1 mM and 60 min, respectively. The upward curvature in the DBC and PZQ curves (RA > 1, Fig. 2) reveals the lytic effect of these drugs. Re^{PROT}, the effective drug/lipid ratio in the membrane for maximal protection, calculated from P^{GHOST} values and Eq. (1) is 0.012, 0.03 and 0.14 for TFP, DBC and PZQ, respectively. These ratios will be discussed later on, together with hemolytic *Re* values.

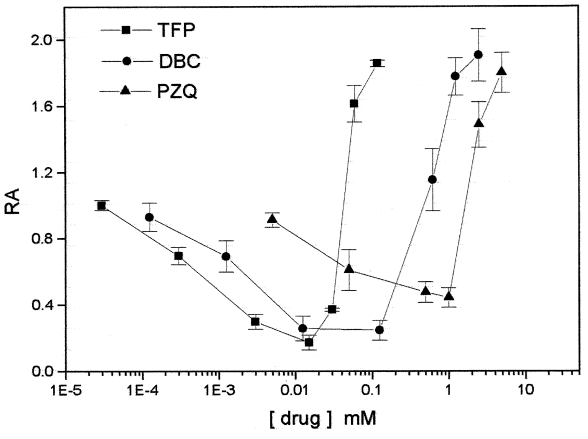

Fig. 2. Drug-induced protection of erythrocyte lysis under hypotonic conditions: for TFP (■), pH 7.4, incubation time = 10 min; for DBC (●), pH 6.8, incubation time = 30 min; for PZQ (▲), pH 7.4, incubation time = 90 min. Ht = 0.14%, hypotonic (66 mM NaCl) 5 mM PBS, room temperature.

Fig. 3 presents the hemolytic curves obtained in *isotonic* medium (150 mM NaCl in PBS buffer) for TFP, DBC and PZQ. At low drug concentrations the membrane incorporates the amphiphiles without losing its integrity, and after membrane saturation, the addition of a small quantity of the drug quickly induces lysis. From the curves in Fig. 3 we obtained C^{SAT} and C^{SOL} values, i.e. the drug concentration for the onset of solubilization of the erythrocyte membrane and complete hemolysis. We point out here the direct correlation observed between P^{GHOST} and hemolytic activity (C^{SAT}, C^{SOL} and also C^{PROT} in Fig. 2). As seen for protection, the most effective drug in inducing lysis was TFP, which also had the highest partition coefficient, while large amounts of PZQ, the drug with the lowest partition into ghosts, are required to disrupt erythrocyte membranes. These data are in agreement with Kanaho's theory that drug entrance into the bilayer is a major step in the hemolytic process [10].

Hemolytic experiments with DBC were conducted at pH 6.8 to prevent the low solubility of DBC at pH 7.4 from limiting its partitioning inside the erythrocyte membranes. We will discuss further that DBC partitioning was not very different at these two pH values.

C^{SAT} and C^{SOL} are plotted as a function of lipid concentration in Fig. 4, to give the straight lines predicted by Eq. (2). The corresponding drug-to-lipid molar ratio in the membrane, *Re,* (Table 3) was readily calculated from the saturation and solubilization lines in Fig. 4. As described above, the straight lines in Fig. 4a–c should intercept the *y* axis at *D*w, the free drug concentration in water that would correspond to the CMC of the amphiphilic molecule in the presence of membranes. For TFP, the obtained *D*w (59 and 94 μM) closely resemble the CMC determined at pH 7.4 (42 μM, Table 1). For DBC at pH 6.8, *D*w values (1.12 and 1.70 mM) were 10 times lower than its CMC and far from its charged

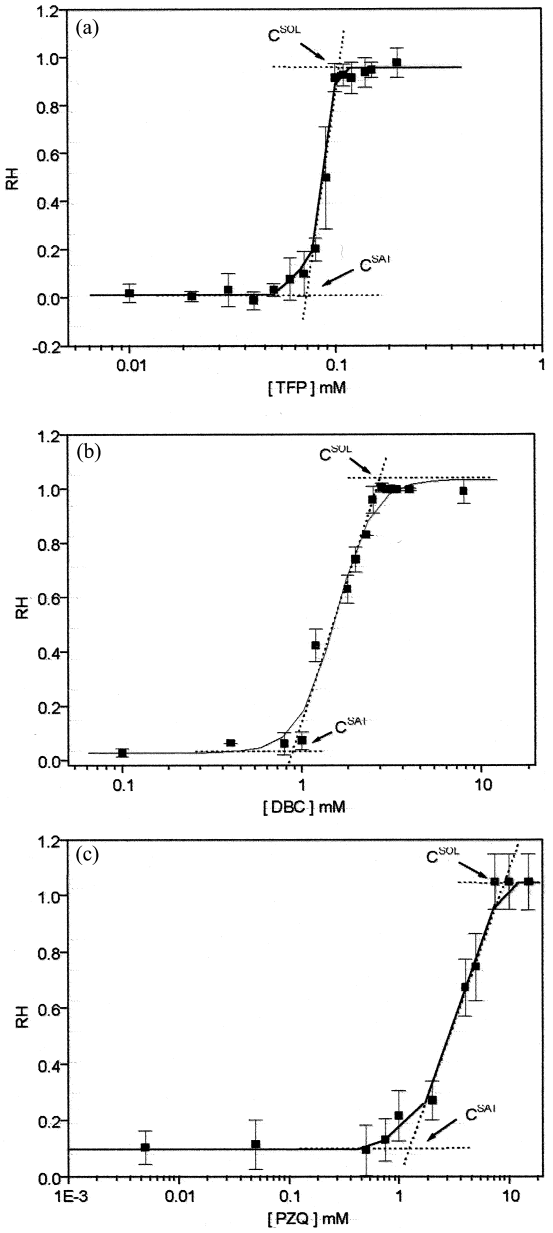

Fig. 3. Hemolytic experiments under isotonic conditions: (a) TFP, pH 7.4, incubation time = 10 min; (b) DBC, pH 6.8, incubation time = 30 min; (c) PZQ, pH 7.4, incubation time = 90 min. Ht = 0.14%, 5 mM PBS, room temperature. C^{SAT} and C^{SOL} (see text) are shown.

form water solubility (Table 1). Dw values came close to zero for PZQ, as if the drug concentration in the aqueous environment were negligible.

TFP showed surfactant-like behavior [12] since its CMC was low (Table 1), indicating a high degree of intermolecular (TFP–TFP) hydrophobic interactions. The aggregate properties of TFP corroborate to its lytic activity since erythrocyte lysis occurs as membrane phospholipids are released to the forming mixed micelles [12]. Keeping this consideration in mind, and going back to DBC, we see that the Dw values (Table 2) are very different from the CMC (10.7 mM at pH 6.8, Table 1) of the anesthetic, indicating that the amphipathic DBC induces lysis in its monomer form. The antihelminthic PZQ is not a surfactant molecule and does not form micelles, but it forms another (solid) phase in the system at concentrations above C^{SOL}.

Table 3 lists C^{SAT}, C^{SOL}, Re and Dw values, obtained for TFP, DBC and PZQ. Re was determined for saturation (Re^{SAT}) and solubilization (Re^{SOL}) experiments, according to [26–28].

We see that a 0.43:1 TFP:lipid ratio (Re^{SAT}) leads to membrane saturation. Above this point, erythrocyte phospholipids probably move to the hydrophobic micelle environment, causing membrane disruption [12] and solubilization is achieved at approximately 1.5:1 TFP:lipid molar ratio (Re^{SOL}). These ratios are in good agreement with those obtained with other amphiphilics like Triton X-100 (0.7:1 and 3:1) lytic effect on egg phosphatidylcholine vesicles [27,44] and erythrocytes [45]. Concerning DBC, Re^{SAT} is 33:1 and Re^{SOL} is 51:1. These very high drug:lipid ratios do not represent a real situation since it is not possible to keep the membrane arrangement with 33 or 51

Table 3
Effective drug/lipid molar ratios[a] and related parameters in the lysis of erythrocytes by TFP, DBC and PZQ

	TFP	DBC	PZQ
C^{SAT} (mM)	0.068	1.4	1.8
C^{SOL} (mM)	0.104	2.8	13.7
Re^{SAT}	0.43	32.9	163
Re^{SOL}	1.45	50.7	765
Dw (μM)[b]	59	1117	~0
K_b (M^{-1})	4783	892	9.9×10^9
P	7268	1356	1.5×10^{10}

[a]According to [26]; experimental conditions given in Fig. 3.
[b]Taken from the saturation curves in Fig. 4.

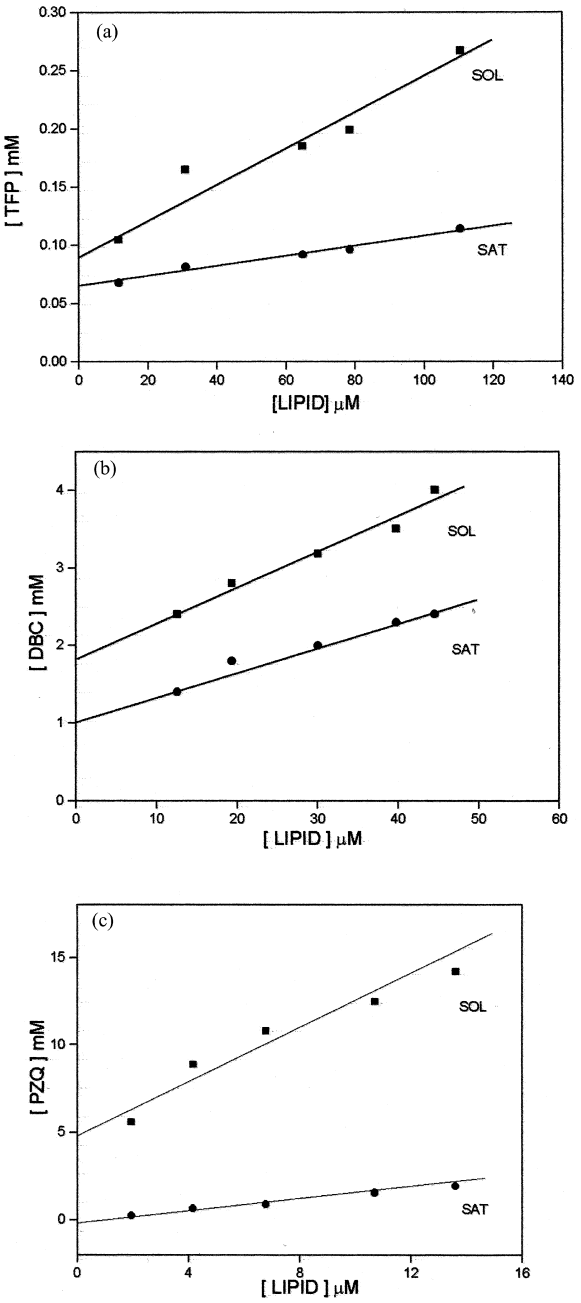

Fig. 4. Effective drug/lipid molar ratio for membrane saturation and solubilization: (a) TFP; (b) DBC; (c) PZQ. C^{SAT} (●) and C^{SOL} (■) were plotted as a function of erythrocyte lipid concentration. *Re* values were taken from the slope of the straight lines.

DBC molecules per lipid molecule. From this point of view, it seems to us that Lichtenberg's treatment [26] can be a useful tool to describe the hemolytic activity of surfactant-like compounds such as TFP, but not of DBC. The high CMC of dibucaine does not characterize it as a surfactant. In the case of PZQ, whose behavior is much more distant from that of a surfactant, the values of Re^{SAT} and Re^{SOL} are completely unreasonable (163:1 and 765:1, respectively), at least concerning drug:lipid molar ratios in the membrane (*Re*).

Following this approach a little further, and assuming ideal partitioning of lipid and drugs in dilute aqueous media [26,29], we can obtain the drug–membrane binding constants (K_b) from the saturation straight lines of Fig. 4 according to Eq. (4):

$$Re^{SAT} = K_b \cdot Dw/(1 - K_b \cdot Dw). \quad (4)$$

The K_b value for TFP between erythrocytes/water was 4.8×10^3 M^{-1} (Table 3). Since K_b and P are related (Eq. (3)), for trifluoperazine, $P =$ 7268. The K_b value obtained for DBC between erythrocytes/water was 8.9×10^2 M^{-1} (Table 3) corresponding to a partition coefficient of 1356. For PZQ, the K_b obtained between erythrocytes/water was quite unreasonable (9.9×10^9 M^{-1} or $P = 1.5 \times 10^{10}$), probably distorted by the very low Dw value, and non-ideal partitioning [37], that does not allow PZQ to be studied by Lichtenberg's treatment.

The K_b determined here for TFP and DBC give overestimated P values in comparison to P^{GHOST} (Table 2) determined by phase separation. Nevertheless, the values for DBC showed better agreement than those for TFP. The hemolytic activity of DBC is mainly determined by its membrane partitioning, while the activity of TFP reflects partition and aggregate phenomena, leading to an overestimate of K_b from the hemolytic experiments (Table 3).

If Lichtenberg's approach seems inappropriate for the three drugs, let us take another look at the drug:lipid ratios for hemolysis, applying

P^{GHOST} and Eq. (1) to calculate Re^{SAT} and Re^{SOL}. The calculated drug:lipid molar ratio inside the membrane for saturation and solubilization, Re^{SAT} and Re^{SOL}, will be 0.06 and 0.09:1 for TFP in the experiment of Fig. 3, with a Ht = 0.14%. The low ratios indicate that TFP-induced hemolysis is not the result of membrane saturation with the drug but is caused by the association of partitioning with achievement of TFP critical micelle concentration. Now for DBC Re^{SAT} and Re^{SOL} will be 0.34 and 0.69:1, respectively, and these ratios seem quite reasonable for true saturation of the membrane phase [46]. Even PZQ, with its small water solubility and non-ideal partitioning, reveals Re^{SAT} and Re^{SOL} values of 0.25 and 1.89:1, indicative of real membrane saturation.

Our data indicate that Lichtenberg's treatment is appropriate for classic surfactant molecules, and only if the concentrations required for hemolysis do not match the CMC range of the surface active compound. For the three drugs studied here the determination of P values by phase separation and *calculations* of drug:lipid molar ratios give more reasonable results. It seems that hemolysis occurs with membrane destabilization due to lipid sequestration from the bilayer caused by the amphiphilic exchange between water (monomer), membrane and also micelle (in the case of TFP) phases; this last exchange facilitates the process as it enhances lipid sequestration from the bilayer, explaining the hemolytic action of TFP at very low (0.06–0.09%) drug:lipid molar ratios. Moreover, the necessity for significant exchange rates justifies the low hemolytic efficiency of PZQ despite its hydrophobic nature.

Protection against hypotonic hemolysis always occurred at lower drug:lipid ratios than Re^{SAT} and Re^{SOL} calculated by this alternative method, for the three drugs. Re^{PROT} values strongly support the accuracy of this analysis.

3.3. Real charged/uncharged ratios for TFP and DBC at physiologic pH

We have mentioned that the low water solubility of PZQ restricts its partition into membranes, explaining the huge amounts of drugs required for hemolysis. But how to explain the higher P values and hemolytic effect of TFP compared to DBC?

In previous studies we reviewed the effect of different partitioning of ionizable local anesthetics [8] and TFP [12] at their ionization constants. It was shown that whenever the binding of charged/uncharged forms is different, it will imply a pK shift of the partitioning compound. Trifluoperazine, for example, has its ionization constant downshifted from 8.1 to 7.6 in the presence of egg PC multilamellar vesicles [12].

Table 4 shows quite different P values for TFP charged/uncharged species, with the neutral form partitioning better ($P_{:}^{LIP} = 2463$) than the protonated, less hydrophobic species ($P_{+}^{LIP} = 812$). For DBC, the $P_{:}^{LIP}$ value was less than two times greater than the P_{+}^{LIP} value (Table 4) and pK_{app},

Table 4
Partition coefficients for charged and uncharged TFP and DBC species between liposomes and water. Ionization constants determined in water (pK) and calculated in the presence of membrane (pK_{app}) and average partition coefficient at pH 7.4 ($P_{average}$) calculated using pK_{app}

Drug	P_{+}^{LIPa}	$P_{:}^{LIPb}$	pK	pK_{app}^{c}	$P_{average}^{d}$
TFP	812 ± 198	2463 ± 274	8.1[e]	7.62	1432
DBC	1790 ± 545	2614 ± 488	8.3[f]	8.13	1919

[a] Determined in 20 mM acetate buffer, pH = 5.0.
[b] Determined in 20 mM carbonate buffer, pH = 10.5.
[c] Calculated from Eq. (5) for Ht = 0.14% (12 μM membrane lipid concentration).
[d] Calculated by Eq. (7) for pH 7.4, using pK_{app} instead of pK in the determination of the base/acid molar ratio (Eq. (6)).
[e] According to [35].
[f] According to [36].

the apparent ionization constant in the presence of membranes, calculated according to [46]:

$$pK_{app} = pK - \log[(P_{:} V_m + V_w) / (P_{+} \cdot V_m + V_w)] \quad (5)$$

was found to be 8.1 in the presence of 12 μM lipids (Ht = 0.14%, Table 3), revealing a downshift of approximately 0.2 pH units. At higher phospholipid concentrations, calculated pK_{app} was the same.

Using pK_{app} in the Henderson–Hasselbalch equation [Eq. (6)], one can calculate the neutral (:) to charged (+) molar ratio at pH 7.4:

$$pH = pK_{app} + \log[base]/[acid]. \quad (6)$$

The neutral/charged molar ratio (Z) for TFP and DBC at pH 7.4 is 0.67:1 and 0.19:1. As a consequence, we can say there is no predominant form of TFP at pH 7.4, while the charged DBC is always the major species under physiologic conditions, with or without membrane.

Now, taking into account the $P_{:}$ and P_{+} values and Z at pH 7.4, an average partition coefficient ($P_{average}$) can be calculated, as shown by Eq. (7):

$$P_{average} = \frac{P_{+} + (P_{:} Z)}{1 + Z}. \quad (7)$$

The calculated values of $P_{average}$, obtained from $P_{:}$ and P_{+} values of TFP and DBC forms (measured at pH 5.0 and 10.5, respectively) between liposomes and water, are listed in Table 4. For TFP $P_{average}$ is 1432 [12].

For DBC in multilamellar liposomes, the calculated $P_{average}$ value was 1919, reflecting the contribution of both charged (84%) and uncharged (16%) forms to its partition at pH 7.4, and showed a very good correlation with the experimental value (P^{LIP} = 1884, Table 2). At pH 6.8, used for the hemolytic experiments for DBC, $P_{average}$ was 1827, i.e. very close to that of DBC at pH 7.4.

$P_{average}$ indicates that partitioning is higher than one would expect by ignoring the pK shift, and hemolytic experiments support this analysis. Hydrophobic membrane interaction is important for the interpretation of the hemolytic effects of the three drugs studied. This explains the direct correlation between the hydrophobicity and biological effects of phenothiazine compounds [11,16,19] and local anesthetics [47].

Besides, $P_{average}$ values reveal that TFP and DBC have similar hydrophobicity at pH 7.4 in phospholipid membranes and their quite different effect on erythrocyte membranes must reflect a different mechanism of action for TFP involving micelle formation and its stronger interaction to erythrocyte's membrane proteins and DBC.

4. General discussion

This work comparatively describes the biphasic (protective/inductive) effect of TFP, DBC and PZQ on mouse erythrocyte hemolysis and elucidates differences in the hemolytic pathway developed by each drug.

The protective effect promoted by the three drugs seems to be triggered by the monomer insertion into the membrane and is directly related to the drugs' hydrophobicity. Protection occurs at low drug:lipid molar ratios (0.01–0.14:1), lower than those required for membrane saturation and solubilization.

The hemolytic effect reveals more details about the mechanism of lysis of each drug. Hemolysis is proportional to the drugs' hydrophobicity (P values) but TFP acts as a surfactant and above CMC it solubilizes membranes [12], while the hemolytic effects of DBC and PZQ are not correlated with the formation of micelles and hemolysis occurs at monomer concentration.

By applying classical treatments for the study of the interaction between surface-active compounds and lipid vesicles [26] to the hemolytic curves, it was possible to calculate drug:lipid molar ratios for the onset (Re^{SAT}) and complete hemolysis (Re^{SOL}). This approach also permitted the calculation of P values from the hemolytic curves, i.e. between whole erythrocytes and water. The results were coherent for TFP, except for the high K_b and P values, but did not seem to be ap-

propriate (very high *Re* values) for the interpretation of DBC and PZQ interaction with erythrocyte membranes.

Instead, the knowledge of P^{GHOST} values allowed the direct calculation of the *Re* values, with better results. Membrane saturation and solubilization occurred at low *Re* values for TFP (0.06 and 0.09), because C^{SAT} and C^{SOL} were close to its CMC while for DBC and PZQ a real membrane saturation (at approx. 0.30:1 drug:lipid) was reached before hemolysis takes place.

We conclude that Lichtenberg's treatment is not always appropriate to interpret surfactant: membrane interaction (even with surface active compounds like TFP). Rather, calculation of the effective drug:lipid molar ratios, based on previously determined *P* values seems to give more reliable results.

The amount of uncharged and charged TFP and DBC species at pH 7.4 was analyzed, taking into account the differences between neutral (:) and protonated (+) binding to membranes and its effect on the real ionization constant, pK_{app} [8,46]. $P_{average}$, the mean partition coefficient at pH = 7.4, was estimated and presented good agreement with the experimental data. For trifluoperazine, $P_{average}$ received an important contribution from the uncharged species, while for DBC there was no important contribution of uncharged species to membrane interaction, explaining the higher hydrophobicity of TFP at pH 7.4 compared to DBC.

This work presents the first report of the effect of PZQ on erythrocyte membranes, showing some of its particular features. Low solubility limits PZQ entrance in the bilayer, configuring a slow partition equilibrium (approx. 60 min between erythrocytes and water) and the need of high quantities of total drug to induce lysis (in order to obtain appropriate Re^{SAT}).

Although hemolysis was always committed to the drug's hydrophobicity, the mechanisms of action of the three drugs were also determined by the aggregate properties of TFP and the restricted water solubility of PZQ. The rate of lipid removal from the bilayer was very high for TFP and hemolysis occurred before a real saturation of the membrane took place; this saturation was in fact observed for DBC and PZQ.

The results presented here should be of help in the understanding of the molecular mechanisms involved in drug-induced hemolysis and in the development of new amphipathic drugs (those which site of action is at the membrane level) with low hemolytic activity.

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